



Original Article

## Fresh garlic extract inhibits *Staphylococcus aureus* biofilm formation under chemopreventive and chemotherapeutic conditions

Panan Ratthawongjirakul\* and Vorraruthai Thongkerd

Department of Transfusion Medicine and Clinical Microbiology, Faculty of Allied Health Sciences,  
Chulalongkorn University, Pathum Wan, Bangkok, 10310 Thailand.

Received: 21 July 2015; Accepted: 25 December 2015

### Abstract

*Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) are the leading aetiological pathogens of nosocomial infections worldwide. These bacteria form biofilms on both biotic and abiotic surfaces causing biofilm-associated infections. Within the biofilm, these bacteria might develop persistent and antimicrobial resistant characteristics resulting in chronic infections and treatment failures. Garlic exhibits broad pharmaceutical properties and inhibitory activities against *S. aureus*. We investigated the effects of aqueous fresh garlic extract on biofilm formation in *S. aureus* ATCC25923 and MRSA strains under chemopreventive and chemotherapeutic conditions. The viable bacteria and biofilm levels were quantified through colony count and crystal violet staining, respectively. The use of fresh garlic extract under both conditions significantly inhibited biofilm formation in *S. aureus* strains ATCC25923 and MRSA. Garlic could be developed as either a prophylactic or therapeutic agent to manage *S. aureus* biofilm-associated infections.

**Keywords:** fresh garlic extract, biofilms, MRSA, *Staphylococcus aureus*

### 1. Introduction

*Staphylococcus aureus* is the leading causative pathogen of nosocomial infections worldwide. It has been estimated that 50 to 70% of nosocomial *S. aureus* infections result from methicillin-resistant *Staphylococcus aureus* (MRSA) (Palavecino, 2004; Lodise and McKinnon, 2005). MRSA strains are resistant to penicillinase-stable penicillins mainly based on the presence of the *mecA* gene, which encodes a novel penicillin-binding protein-2a (Chambers, 2001). MRSA strains have been implicated in many difficult-to-treat infections, reflecting the antibiotic-resistant characteristics of these bacteria. Therefore, patients infected with MRSA might be at increased risk for delayed treatment, morbidity, and mortality (Lodise and McKinnon, 2005).

*S. aureus* are characterised by the formation of biofilms on biomaterials, damaged tissues, and most commonly on indwelling medical devices, causing biofilm-associated infections (Otto, 2008; Makino *et al.*, 2013). These infections are becoming more common and occur widely, reflecting the increased use of indwelling medical devices over the past few decades (Crnich and Maki, 2002). In clinical settings, these organisms persist in sessile environments, such as in biofilms, resulting in chronic infections (Gowrishankar *et al.*, 2012). Over a half of *S. aureus*, including MRSA, have the ability of biofilm formation in various levels (Indrawattana *et al.*, 2013; Rezaei *et al.*, 2013). Furthermore, *S. aureus* also gain increased resistance to antimicrobial agents through biofilm formation as a bacterial survival strategy (Donlan and Costerton, 2002; Hall-Stoodley *et al.*, 2004). In addition, nosocomial MRSA infection is based on non-specific mechanism of resistance, which biofilm formation is involved (Otto, 2008). Consequently, biofilms formed by MRSA might become more resistant to most available antimicrobial agents, resulting in treatment failures.

\* Corresponding author.

Email address: panan\_etc@yahoo.com

A biofilm is “a matrix enclosed bacterial population that irreversibly adheres to each other and to a substratum” (Costerton *et al.*, 1995). Bacteria initiate biofilm formation in response to environmental factors, such as nutrient and oxygen availability (Thomas and Lehman, 2006). In Staphylococci, the intercellular adhesin (*ica*) operon encodes the extracellular polymeric biofilm matrix, referred to as polysaccharide intercellular adhesin (PIA) (Gotz, 2002). The nature of the biofilm structure confers inherent antimicrobial resistance. Biofilm-associated infections are 10 to 1,000 times more resistant to conventional antimicrobial agents (Thien-Fah and O’Toole, 2001). Several antibiotics have been shown to significantly affect *S. aureus* planktonic cells, but not the bacteria in the biofilm. Rifampicin rapidly killed planktonic MRSA but did not significantly reduce biofilm growth, and the biofilm remained established on the Silastic surface (Jones *et al.*, 2001). Moreover, vancomycin, quinupristin/dalfopristin, and linezolid showed little effect on the viability of bacteria in biofilms *in vitro*, and efficient killing of bacterial cells in biofilms was observed only at higher concentrations (50, 500 and 1,000 µg/ml) of these individual agents (El-Azizi *et al.*, 2005). The occurrence of penicillin-resistant biofilm producing *S. aureus* varied from 86.3 to 88%, followed by oxacillin resistance (72.7 to 82%), cefazolin resistance (62.6 to 63.6%) and ciprofloxacin resistance (53.7 to 54.5%). Furthermore, 53.7% of biofilm-producing MRSA isolates were also resistant to ciprofloxacin while 17.6% of non-biofilm-producing MRSA were ciprofloxacin resistant (Agarwal and Jain, 2012). The antimicrobial resistance of *S. aureus* biofilm-associated infections has become a global problem, resulting in the wide spread of nosocomial infections.

Garlic (*Allium sativum* L.) and garlic extracts have been previously demonstrated as effective in inhibiting the growth of different bacterial pathogens, including Staphylococci and MRSA (Tsao and Yin, 2001; Tsao *et al.*, 2003; Rattanachaiakunsopon and Phumkhachorn, 2009; Houshmand *et al.*, 2013). Chemical analysis of garlic oil extract showed that 54.5% of the total sulphides comprise diallyl monosulphide, diallyl disulphide, diallyl trisulphide and diallyl tetrasulphide, and the minimum inhibitory concentration of whole garlic oil extract against MRSA was 32 µl/ml, whereas the MICs for the individual sulphide compounds were 32, 12, 8 and 2 µl/ml, respectively (Lawson *et al.*, 1991). Allicin, the active compound in fresh garlic extract, also inhibited the proliferation of MRSA in a dose-dependent manner (Cutler and Wilson, 2004). Recently, garlic extract has been shown to diminish the biofilm formation of some microbes, such as *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *Candida albicans* (Perez-Giraldo *et al.*, 2003; Bjarnsholt *et al.*, 2005; Shuford *et al.*, 2005). The aim of the present study was to investigate the effects of aqueous fresh garlic extract on biofilm formation in *S. aureus* and MRSA under two *in vitro* culture conditions, including a chemopreventive setting, in which the bacteria were grown in the presence of garlic, and a chemotherapeutic setting, in which garlic was

added after the formation of the mature biofilm.

## 2. Materials and Methods

### 2.1 Bacterial strains

*S. aureus* strain ATCC25923 and one clinical MRSA strain isolated from the Microbiology Unit of Ramathibodi Hospital, Bangkok, Thailand, were used in the present study. The MRSA isolate was detected with cefoxitin (30 µg, Oxoid, Hampshire, UK) using the disk diffusion method, according to the Clinical and Laboratory Standards Institute (CLSI) document M100-24 (CLSI, 2013). The bacteria were grown on tryptic soy agar (TSA, Oxoid, Hampshire, UK) and incubated at 37°C for 24 h.

### 2.2 Fresh garlic extract preparation

To prepare fresh garlic extract (FGE), 200 g of garlic cloves were peeled and roughly crushed in sterile distilled water. A final concentration was adjusted to 20 mg/ml. The extract was centrifuged at 6,000 rpm for 10 min, and the supernatant was filtered through a 0.45-µm membrane. The stock solution of FGE was stored at -80°C until further use.

### 2.3 Minimum inhibitory concentration (MIC) tests

The MIC of FGE was determined using the broth dilution technique in accordance with the CLSI document M07A9 (CLSI, 2013). The FGE stock solution was diluted in Mueller-Hinton broth (Oxoid, Hampshire, UK) to final concentrations of 128, 256, 512, 1,024, 2,048, 4,096, 8192, and 16,384 µg/ml. After culturing for 24 h, the *S. aureus* ATCC 25923 and MRSA bacterial colonies were suspended in 0.85% (w/v) sterile normal saline, and the final bacterial concentrations were adjusted to a 0.5 McFarland density standard (~1-2x10<sup>8</sup> CFU/ml). The bacterial suspensions were added to Mueller-Hinton broth containing FGE at one of the concentrations specified above. The tubes were incubated at 37°C for 16-18 h. The MIC was reported as the lowest concentration of the compound required to visibly inhibit the growth of bacteria.

### 2.4 Screening of biofilm production

Biofilm formation in both *S. aureus* ATCC25923 and MRSA was preliminarily evaluated through cultivation on Congo red agar (CRA) (Croes *et al.*, 2009). Briefly, CRA plates were prepared using TSA containing 0.08% (w/v) Congo red (Sigma, Missouri, USA) and 5% (w/v) sucrose. Plates were incubated at 37°C for 24 h. The following descriptions were used to interpret the colony phenotypes: black colonies with rough, dry surfaces were considered positive for slime (biofilm) production, whereas red colonies with shiny surfaces were considered negative for slime production.

## 2.5 Biofilm formation assay

Biofilm formation assay was established as previously described (Stepanovic *et al.*, 2000) with some modifications. After culturing for 24 h, the *S. aureus* ATCC25923 and MRSA bacterial colonies were suspended in tryptic soy broth (TSB, Oxoid, Hampshire, UK). For chemopreventive conditions, the bacterial suspension was inoculated into flat-bottomed 96-well polystyrene microtiter plates (Nunc, New York, USA). The final bacterial concentration was adjusted to  $10^6$  CFU/ml in a total volume of 100  $\mu$ l. FGE was subsequently added to the wells, and the final concentration was adjusted to sub-MIC. The plates were incubated at 37°C for 24 h. For chemotherapeutic conditions, 100  $\mu$ l of the bacterial suspension was inoculated into flat-bottomed 96-well polystyrene microtiter plates at a final bacterial concentration of  $10^6$  CFU/ml. The plates were incubated at 37°C for 24 h to facilitate mature biofilm formation. Subsequently the bacterial culture solutions were discarded, and the adherent biofilms were washed twice with PBS. One hundred microliters of fresh TSB was added to the wells, followed by the addition of FGE at a sub-MIC final concentration. The plates were incubated at 37°C for 24 h. Bacterial biofilms without FGE treatment were used as controls in parallel experiments. Un-inoculated FGE-free and un-inoculated FGE-supplemented media were established to define the background OD values. Three replicate wells for each treatment were performed.

## 2.6 Colony counting

The viable bacteria in the biofilms were examined by determining the number of CFU/ml. After incubation, the bacterial culture solutions were discarded, and the wells were thoroughly washed three times with PBS. One hundred microliters of TSB was added to the wells, and the adherent biofilms were scraped using sterile plastic tips. The suspensions were serially diluted 10-fold in TSB and plated in duplicate onto TSA plates, followed by incubation at 37°C for 24 h. Plates with more than 25 but less than 250 colonies were considered.

## 2.7 Quantification of biofilm

The biofilm levels were quantitated using a crystal violet staining technique. After incubation, the bacterial culture solutions were discarded, and the wells were thoroughly washed three times with PBS. The plates were subsequently dried at 60°C for 30 min. The adherent biofilms in each well were stained with 125  $\mu$ l of a 0.1% (v/v) solution of crystal violet in water at room temperature for 15 min. The plates were rinsed three times with water by submerging in a tub of water and tapping vigorously on a paper towel to completely remove all excess cells and dye. The plates were dried at room temperature overnight. Approximately 125  $\mu$ l of ethanol:acetic acid (95:5, v/v) was added to each well to solubilise the crystal violet. The plates were incubated at

room temperature for 15 min. The solubilised crystal violet was transferred to new flat-bottomed 96-well polystyrene microtiter plates, and the absorbance at 570 nm was measured using a spectrophotometer. The mean OD<sub>570</sub> values for the control and tested wells were subtracted from the mean OD<sub>570</sub> values obtained from the un-inoculated FGE-free and un-inoculated FGE-supplemented wells, respectively.

## 2.8 Statistical analysis

The data represent the average of three experiments performed in triplicate. For statistical analysis, Student's t-test was used to determine differences between *S. aureus* and MRSA. One-way analysis of variance (one-way ANOVA) was used to determine differences among control FGE-untreated and experimental FGE-treated groups. A multiple comparison between statistically significant sample pairs was determined using the Fisher's least significant difference (LSD) test. Probability values of  $P < 0.05$  were considered significant.

## 3. Results

The inhibitory activity of FGE against *S. aureus* ATCC25923 and one clinical MRSA isolate was examined *in vitro* using the broth dilution method. FGE inhibited the growth of both strains with an MIC of 8,192  $\mu$ g/ml. The sub-MIC was considered as one-fold concentration lower than the MIC, i.e., 4,096  $\mu$ g/ml. The bacteria were subsequently cultured on CRA to detect biofilm formation. Both strains were likely to produce biofilm according to the colony appearance shown on CRA. Black colonies with a rough, dry surface were observed in *S. aureus* ATCC25923 and MRSA after incubation for 24 h (Figure 1).

Next, we evaluated the effect of a sub-MIC concentration of FGE on biofilm formation in *S. aureus* ATCC25923 and MRSA under both chemopreventive and chemotherapeutic conditions. The colony count technique was used to quantitate the number of viable bacteria in the scraped biofilms. FGE significantly reduced the bacterial counts in the groups treated under chemopreventive and chemotherapeutic conditions compared with the control FGE-untreated groups. The *S. aureus* ATCC25923 bacterial counts (as expressed as log<sub>10</sub>



Figure 1. Colony appearance on CRA. The appearance of black, rough and dry colonies on CRA represents biofilm formation in *S. aureus* ATCC25923 (A and C) and MRSA (B).

CFU/ml) in the control FGE-untreated, chemopreventive, and chemotherapeutic groups were  $2.217 \pm 0.625$ ,  $0.039 \pm 0.034$ , and  $0.109 \pm 0.084$ , respectively (Figure 2A). The *S. aureus* bacterial counts in both the chemopreventive and chemotherapeutic groups were significantly lower than those in the control FGE-untreated group ( $P < 0.01$ ). However, the difference in the bacterial count between the chemopreventive and chemotherapeutic groups was not significant ( $P > 0.05$ ). Similar bacterial counts were also observed for MRSA. The bacterial counts in the control FGE-untreated, chemopreventive, and chemotherapeutic MRSA groups were  $1.233 \pm 1.202$ ,  $0.135 \pm 0.131$ , and  $0.155 \pm 0.119$ , respectively (Figure 2B). The bacterial counts in both the chemopreventive and chemotherapeutic MRSA groups were significantly lower than those in the control FGE-untreated group ( $P < 0.05$ ). Notably, no significant difference in the bacterial counts was observed between chemopreventive and chemotherapeutic conditions ( $P > 0.05$ ). While the bacterial counts between *S. aureus* and MRSA

in the control FGE-untreated and chemotherapeutic groups were similar ( $P > 0.05$ ), the *S. aureus* bacterial counts were significantly lower than the MRSA in the chemopreventive group ( $P < 0.05$ ).

The bacterial biofilms at the bottom of the well were stained with crystal violet, and the absorbance was measured at 570 nm. The level of biofilm formation was expressed as the mean OD<sub>570</sub> value obtained for the sample subtracted by the mean OD<sub>570</sub> value obtained for the blank. Figure 3 shows the mean absorbance obtained for *S. aureus* ATCC25923 and MRSA biofilms under both chemopreventive and chemotherapeutic conditions. The mean absorbance values obtained for the control FGE-untreated, chemopreventive, and chemotherapeutic *S. aureus* groups were  $0.546 \pm 0.396$ ,  $0.180 \pm 0.250$ , and  $0.380 \pm 0.185$ , respectively (Figure 3A), whereas the mean absorbance values obtained for the untreated, chemopreventive and chemotherapeutic MRSA groups were  $0.403 \pm 0.573$ ,  $0.104 \pm 0.051$ , and  $0.265 \pm 0.040$ , respectively (Figure 3B). Thus,

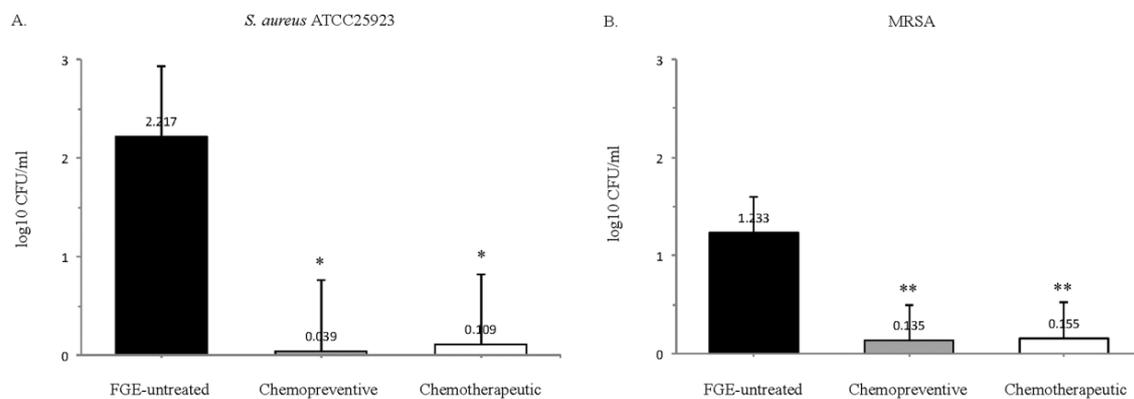


Figure 2. Bacterial counts obtained from the scraped biofilms under chemopreventive and chemotherapeutic conditions. The viable bacteria recovered from the scraped *S. aureus* ATCC25923 (A) and MRSA (B) biofilms after 24 h of incubation are expressed as log<sub>10</sub> CFU/ml. The control FGE-untreated biofilm, and the chemopreventive, and chemotherapeutic conditions are represented in black, grey, and white, respectively. The means and standard deviations were calculated from three independent experiments. The bars with one and two asterisks are significantly different from the controls (ANOVA;  $P < 0.01$  and  $P < 0.05$ , respectively).

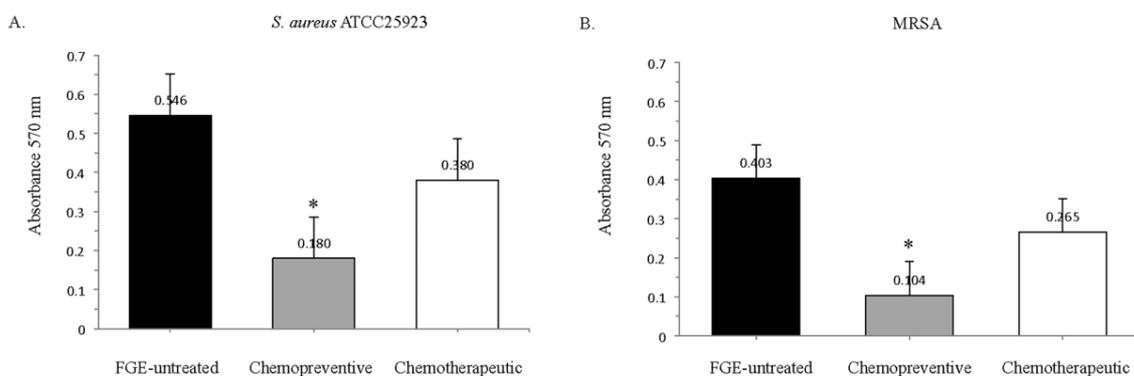


Figure 3. Biofilm quantification under chemopreventive and chemotherapeutic conditions. The level of *S. aureus* ATCC25923 (A) and MRSA (B) biofilm formation after 24 h of incubation was quantified using crystal violet staining. The absorbance was measured at 570 nm. The control FGE-untreated biofilm and the chemopreventive and chemotherapeutic conditions are represented in black, grey, and white, respectively. The means and standard deviations were calculated from three independent experiments. The bars with an asterisk are significantly different from the controls (ANOVA;  $P < 0.05$ ).

under chemopreventive conditions, FGE significantly reduced the levels of biofilm formation in both *S. aureus* ATCC25923 and MRSA compared with the control FGE-untreated groups ( $P < 0.05$ ). In contrast, the statistical analysis showed no significant reduction in *S. aureus* ATCC25923 and MRSA biofilm formation under chemotherapeutic conditions ( $P > 0.05$ ). Levels of biofilm formation in *S. aureus* were significantly higher than in MRSA under the chemopreventive group ( $P < 0.05$ ) and chemotherapeutic group ( $P < 0.01$ ), whereas no significant difference was found in the two strains in the control FGE-untreated groups ( $P > 0.05$ ).

#### 4. Discussion

The long history of the medicinal uses of garlic has been well documented. Indeed garlic exhibits biological activities, such as anti-inflammatory, anti-thrombotic, anti-atherosclerotic, serum lipid lowering, anti-cancer and anti-microbial activities (Harris *et al.*, 2001; Bayan *et al.*, 2014). Garlic also exhibits inhibitory activity against several common human bacterial pathogens, such as *Staphylococcus aureus*, *Escherichia coli*, *Staphylococcus hemolyticus*, *Klebsiella* spp., *Shigella dysenteriae*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Mycobacterium tuberculosis* and *Candida albicans* (Hall-Stoodley *et al.*, 2004; Nidadavolu *et al.*, 2012; Viswanathan *et al.*, 2014). Compared with garlic powder extract, FGE has greater effects on the morphology and growth inhibition of *C. albicans* (Lemar *et al.*, 2002). In the present study, we showed that FGE exhibited significant inhibitory activity against *S. aureus* ATCC25923 and MRSA. Allicin is the active compound in garlic that impacts bacterial viability (Cavallito and Bailey, 1944). The antimicrobial properties of allicin have been reported in many *in vitro* studies (Di Paolo and Carruthers, 1960; Bouchara *et al.*, 1986; De Pauw *et al.*, 1995). Interestingly, allicin exhibited equal LD<sub>50</sub> on both antibiotic-sensitive and antibiotic-resistant bacteria (Chowdhury *et al.*, 1991). In the present study, we observed that *S. aureus* ATCC25923 and MRSA were inhibited by a similar concentration of FGE at an MIC of 8,192 µg/ml. Thus, the antimicrobial activity of garlic is based on two principal features. First, the compound must reach the potential target. Considering intracellular targets, the active garlic compound must penetrate the microbial cells. Allicin has been demonstrated to readily diffuse across both artificial and natural phospholipid membranes (Miron *et al.*, 2000). Inside the cell, the antibiotic efficiency of allicin depends on reaching and reacting with targets that are important to the cell. The antimicrobial effect of allicin likely reflects an interaction with the thiol-containing enzymes in microorganisms (Cavallito and Bailey, 1944). At slightly higher concentrations of allicin, other enzymes, such as dehydrogenases or thioredoxin reductases, might be affected and could be lethal to microorganisms (Ankri and Mirelman, 1999). Allicin specifically inhibits acetate kinase and phosphotransacetyl-CoA synthetase, which are bacterial enzymes in the acetyl-CoA-forming system, and this compound also

affects the DNA and protein in *Salmonella Typhimurium* (Feldberg *et al.*, 1988; Focke *et al.*, 1990).

*S. aureus* and MRSA are the most frequent causes of nosocomial infections associated with indwelling medical devices, likely involving bacterial biofilm formation (Otto, 2008; Rewatkar and Wadher, 2013). *S. aureus* biofilm-associated infections are often difficult to treat with antibiotics (Thien-Fah and O'Toole, 2001), reflecting the fact that biofilm-embedded bacteria are 100-1000 times more resistant to antibiotics than their planktonic counterparts (Widmer, 2001). However, alternative approaches to overcome biofilm formation, in lieu of antibiotic treatment, have been investigated and developed (Al-Adham *et al.*, 2003; Balaban *et al.*, 2005). Recently, various preparations of garlic have been used to inhibit the biofilm formation of some bacterial pathogens. Garlic ointment prevented biofilm development in burn wound bacterial pathogens, such as *S. epidermidis*, *P. aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumonia* (Nidadavolu *et al.*, 2012). At a concentration of 2 to 4 mg/ml, FGE demonstrated superior inhibitory effects against mature biofilm formation in *C. albicans* (Shuford *et al.*, 2005). *In vivo* studies in a rabbit model demonstrated that allicin inhibited biofilm formation and enhanced the bactericidal effect of vancomycin against *S. epidermidis* on the implant surfaces of prosthetic joint infections (Zhai *et al.*, 2014). In the present study, we showed that sub-MIC concentrations of aqueous FGE inhibited biofilm formation in *S. aureus* ATCC25923 and MRSA. Compared with the FGE-untreated groups, the viable bacterial colony count obtained from scraped biofilms and the crystal violet absorbance values representing the biofilm levels were significantly reduced under both chemopreventive and chemotherapeutic conditions. Allicin is exclusively responsible for the antimicrobial activity of freshly crushed garlic (Borlinghaus *et al.*, 2014). Thus, based on the results of the present study, we propose that the antibacterial or anti-biofilm activities of garlic against *S. aureus* ATCC25923 and MRSA might primarily reflect the activity of allicin.

We established two *in vitro* culture conditions to investigate the effects of FGE on biofilm formation in *S. aureus* ATCC25923 and MRSA. Under chemopreventive conditions, the bacteria were grown in the presence of garlic. Under chemotherapeutic conditions, garlic was added to the culture after formation of the mature biofilm. We attempted to determine which condition would favor the inhibition of biofilm formation. However, the statistical analysis demonstrated no significant difference in the reduction of biofilm formation between these two conditions. In the chemopreventive setting, FGE treatment resulted in slightly lower bacterial colony counts and crystal violet absorbance values than treatment in the chemotherapeutic setting for both strains. There are several possible explanations for this result, which are not mutually exclusive. In the chemopreventive setting, due to the inhibitory activity of garlic against *S. aureus* (Nidadavolu *et al.*, 2012), FGE might directly inhibit the growth of bacteria in the planktonic phase. *S. aureus* biofilm development involves in 2-step process: initial attach-

ment and subsequent maturation (Otto, 2008). Garlic likely interferes with bacterial adherence, determined as the primary crucial step for biofilm formation. A previous study showed that sub-MIC concentrations of allicin prevented *S. epidermidis* adherence and biofilm formation *in vitro* (Perez-Giraldo *et al.*, 2003), potentially reflecting the reduced level of biofilm formation observed under chemopreventive conditions compared with chemotherapeutic conditions.

Previous studies have investigated the potential molecular mechanisms underlying the garlic-mediated inhibition of bacterial biofilm formation. In Staphylococci, the biofilm comprises a matrix of extracellular polymeric substances called polysaccharide intercellular adhesins (PIAs), which mediate the attachment of bacterial cells and facilitate biofilm development. PIA is a  $\beta$ -1,6-linked *N*-acetylglucosamine synthesised from UDP-*N*-acetylglucosamine through the enzyme *N*-acetylglucosaminyltransferase encoded by the *ica* operon. This operon comprises *icaADBC* biosynthesis genes, which are tightly controlled through numerous regulatory factors. The fifth gene, *icaR*, is a negative regulator of *icaADBC* (Gotz, 2002). Deletion of the *ica* locus genes presumably leads to defects in biofilm formation, intracellular aggregation, and PIA synthesis in *S. epidermidis* (Heilmann *et al.*, 1996), suggesting that the *icaADBC* is necessary for the maturation of bacterial biofilms. Recently, garlic has been shown to interfere with biofilm-associated genes. Quantitative RT-PCR analyses have revealed that allicin down-regulates the expression of *icaA*, and *aap* (accumulation-associated protein) in *S. epidermidis* mature biofilms (Wu *et al.*, 2015). These may represent a related mechanism of garlic in the reduction of biofilm formation observed under chemotherapeutic conditions in this study. However, the convincing inhibitory mechanism of garlic on biofilm-associated genes remains unclear.

Many pathogenic bacteria use the quorum-sensing (QS) system to coordinate bacterial virulence expression, including biofilm development. QS is a highly specific antimicrobial target that is not present in humans (Bhardwaj *et al.*, 2013). Thus, QS might be a promising strategy to control bacterial virulence. Some studies have suggested garlic as a potential QS-interfering compound. DNA microarray analysis revealed that Ajoene, a garlic-derived sulphur-containing compound, specifically inhibited QS-regulated gene expression in *P. aeruginosa* (Jakobsen *et al.*, 2012). Allicin and FGE decreased quorum-sensing signals and biofilm formation and inhibited QS-controlled virulence factors in *P. aeruginosa* (Rasmussen *et al.*, 2005; Lihua *et al.*, 2013). These results suggest that the reduction of biofilm formation in *P. aeruginosa* might reflect the quorum-sensing inhibitory properties of garlic. *S. aureus* utilises autoinducing peptides as a signal in the QS system (Ji *et al.*, 1995). This system is referred to as the accessory gene regulator system, comprising two operons, RNAII and RNAIII, on the *agr* locus (Novick, 2003; Gov *et al.*, 2004). The up-regulation of *agr* has been observed in biofilm-forming *S. epidermidis* (Batzilla *et al.*, 2006). However, a surprising role for *agr* in *S. aureus* biofilm forma-

tion has been reported (Yarwood *et al.*, 2004). In contrast to *S. epidermidis*, *S. aureus* cell dispersal from the biofilm surface has been associated with *agr* up-regulation (Lauderdale *et al.*, 2010). A recent microarray analysis demonstrated that natural compounds, such as Manuka honey, down-regulate *agr* expression in MRSA (Jenkins *et al.*, 2014). Inhibition of the *agr* system attenuates the virulence of *S. aureus*, reducing the progression and persistence of the associated diseases (Bhardwaj *et al.*, 2013). However, whether garlic interferes with *agr* expression and sequentially impacts biofilm formation in *S. aureus* requires further investigation.

Because garlic displays anti-biofilm activity, this compound has been suggested as a potential therapeutic agent for controlling biofilm formation in *S. aureus*. Garlic has been recently applied to medical innovation. Garlic ointment, containing pure garlic powder in petroleum jelly, has been developed as a novel agent to prevent wound pathogen biofilm formation (Nidadavolu *et al.*, 2012). Several strategies to prevent catheter-associated biofilm infections have been developed, including coating the catheter with antimicrobial agents, organoselenium, and silver (Tran *et al.*, 2012; Lajcak *et al.*, 2013; Jamal *et al.*, 2014). Based on the reduction of biofilm formation in *S. aureus* and MRSA through FGE observed in the present study, a garlic-coated catheter could also be developed.

## 5. Conclusions

In summary, we showed that aqueous FGE not only inhibited the growth of *S. aureus* ATCC25923 and MRSA but also reduced biofilm formation in these bacteria under both chemopreventive and chemotherapeutic conditions. However, the molecular mechanism underlying the influence of garlic on bacterial viability and biofilm formation in *S. aureus* requires further in-depth study. The results obtained in the present study suggest that garlic might represent a promising prophylactic or therapeutic candidate for the management of *S. aureus* biofilms.

## References

- Agarwal, A. and Jain, A. 2012. Association between drug resistance and production of biofilm in staphylococci. *Indian Journal of Medicinal Research*. 135, 562-564.
- Al-Adham, I.S., Al-Hmoud, N.D., Khalil, E., Kierans, M., and Collier, P.J. 2003. Microemulsions are highly effective anti-biofilm agents. *Letters in Applied Microbiology*. 36, 97-100.
- Ankri, S. and Mirelman, D. 1999. Antimicrobial properties of allicin from garlic. *Microbes and Infection*. 2, 125-129.
- Balaban, N., Stoodley, P., Fux, C.A., Wilson, S., Costerton, J.W., and Dell'Acqua G. 2005. Prevention of staphylococcal biofilm-associated infections by the quorum sensing inhibitor RIP. *Clinical Orthopaedics and Related Research* 437, 48-54.

- Batzilla, C.F., Rachid, S., Engelmann, S., Hecker, M., Hacker, J., and Ziebuhr, W. 2006. Impact of the accessory gene regulatory system (Agr) on extracellular proteins, *codY* expression and amino acid metabolism in *Staphylococcus epidermidis*. *Proteomics*. 6, 3602-3613.
- Bayan, L., Koulivand, P.H., and Gorji, A. 2014. Garlic: a review of potential therapeutic effects. *Avicenna Journal of Phytomedicine*. 4, 1-14.
- Bhardwaj, A.K., Vinothkumar, K., and Rajpara, N. 2013. Bacterial quorum sensing inhibitors: attractive alternatives for control of infectious pathogens showing multiple drug resistance. *Recent Patents on Anti-infective Drug Discovery*. 8, 68-83.
- Bjarnsholt, T., Jensen, P.O., Rasmussen, T.B., Christophersen, L., Calum, H., Hentzer, M., Hougen, H.P., Rygaard, J., Moser, C., Eberl, L., Hoiby, N., and Givskov, M. 2005. Garlic blocks quorum sensing and promotes rapid clearing of pulmonary *Pseudomonas aeruginosa* infections. *Microbiology*. 151, 3873-3880.
- Borlinghaus, J., Albrecht, F., Gruhlke, M.C., Nwachukwu, I.D., and Slusarenko, A.J. 2014. Allicin: chemistry and biological properties. *Molecules*. 19, 12591-12618.
- Bouchara, J.P., Tronchin, G., and Senet, J.M. 1986. Laminin receptors on *Candida albicans* in vitro. A preliminary study. *Journal of Medical and Veterinary Mycology*. 24, 48-54.
- Cavallito, C. and Bailey, J.H. 1944. Allicin, the antibacterial principle of *Allium sativum*. Isolation, physical properties and antibacterial action. *Journal of the American Chemical Society*. 66, 1944-1952.
- Chambers, H.F. 2001. Methicillin-resistant *Staphylococcus aureus*. Mechanisms of resistance and implications for treatment. *Postgraduate Medicine*. 109, 43-50.
- Chowdhury, A.K., Ahsan, M., Islam, S.N., and Ahmed, Z.U. 1991. Efficacy of aqueous extract of garlic and allicin in experimental shigellosis in rabbits. *Indian Journal of Medical Research*. 93, 33-36.
- Clinical and Laboratory Standards Institute. 2013. M100-S23 Performance standards for antimicrobial susceptibility testing twenty-third informational supplement. Volume 23 (1). Wayne, Pennsylvania, U.S.A.
- Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., and Lappin-Scott, H.M. 1995. Microbial biofilms. *Annual Review of Microbiology*. 49, 711-745.
- Crnich, C.J. and Maki, D.G. 2002. The promise of novel technology for the prevention of intravascular device-related bloodstream infection. II. Long-term devices. *Clinical Infectious Diseases*. 34, 1362-1368.
- Croes, S., Deurenberg, R.H., Boumans, M.L., Beisser, P.S., Neef, C., and Stobberingh, E.E. 2009. *Staphylococcus aureus* biofilm formation at the physiologic glucose concentration depends on the *S. aureus* lineage. *BMC Microbiology*. 9, 229 doi:10.1186/1471-2180-9-229.
- Cutler, R.R. and Wilson, P. 2004. Antibacterial activity of a new, stable, aqueous extract of allicin against methicillin-resistant *Staphylococcus aureus*. *British Journal of Biomedical Science*. 61, 71-74.
- De Pauw, B.E., Raemaekers, J.M., Donnelly, J.P., Kullberg, B.J., and Meis, J.F. 1995. An open study on the safety and efficacy of fluconazole in the treatment of disseminated *Candida* infections in patients treated for hematological malignancy. *Annals of Hematology*. 70, 83-87.
- Di Paolo, J.A. and Carruthers, C. 1960. The effect of allicin from garlic on tumour growth. *Cancer Research*. 20, 431-434.
- Donlan, R.M. and Costerton, J.W. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Review*. 15, 167-193.
- El-Azizi, M., Rao, S., Kanchanapoom, T., and Khardori, N. 2005. In vitro activity of vancomycin, quinupristin/dalfopristin, and linezolid against intact and disrupted biofilms of staphylococci. *Annals of Clinical Microbiology and Antimicrobials*. 4, 2.
- Feldberg, R.S., Chang, S.C., Kotik, A.N., Nadler, M., Neuwirth, Z., Sundstrom, D.C., and Thompson, N.H. 1988. In vitro mechanism of inhibition of bacterial cell growth by allicin. *Antimicrobial Agents Chemotherapy*. 32, 1763-1768.
- Focke, M., Feld, A., and Lichtenthaler, K. 1990. Allicin, a naturally occurring antibiotic from garlic, specifically inhibits acetyl-CoA synthetase. *FEBS Letters*. 261, 106-108.
- Gotz, F. 2002. *Staphylococcus* and biofilms. *Molecular Microbiology*. 43, 1367-1378.
- Gov, Y., Borovok, I., Korem, M., Singh, V.K., Jayaswal, R.K., Wilkinson, B.J., Rich, S.M., and Balaban, N. 2004. Quorum sensing in *Staphylococci* is regulated via phosphorylation of three conserved histidine residues. *The Journal of Biological Chemistry*. 279, 14665-14672.
- Gowrishankar, S., Duncun, M.N., and Karutha, P.S. 2012. Coral-associated bacteria as a promising antibiofilm agent against methicillin-resistant and -susceptible *Staphylococcus aureus* biofilms. *Evidence-Based Complementary and Alternative Medicine*. 2012, 862374. doi:10.1155/2012/862374.
- Hall-Stoodley, L., Costerton, J.W., and Stoodley, P. 2004. Bacterial biofilms: from the natural environment to infectious diseases. *Nature Review Microbiology*. 2, 95-108.
- Harris, J.C., Cottrell, S.L., Plummer, S., and Lloyd, D. 2001. Antimicrobial properties of *Allium sativum* (garlic). *Applied Microbiology and Biotechnology*. 57, 282-286.
- Heilmann, C., Schweitzer, O., Gerke, C., Vanittanakom, N., Mack, D., and Gotz, F. 1996. Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. *Molecular Microbiology*. 20, 1083-1091.

- Houshmand, B., Mahjour, F., and Dianat, O., 2013. Antibacterial effect of different concentrations of garlic (*Allium sativum*) extract on dental plaque bacteria. *Indian Journal of Dental Research*. 24, 71-75.
- Indrawattana, N., Sungkhachat, O., Sookrung, N., Chongsanguan, M., Tungtrongchitr, A., Voravuthikunchai, S.P., Kong-ngoen, T., Kurazono, H., and Chaicumpa, W. 2013. *Staphylococcus aureus* clinical isolates: antibiotic susceptibility, molecular characteristics, and ability to form biofilm. *BioMed Research International*. <http://dx.doi.org/10.1155/2013/314654>.
- Jakobsen, T.H., van Gennip, M., Phipps, R.K., Shanmugham, M.S., Christensen, L.D., Alhede, M., Skindersoe, M.E., Rasmussen, T.B., Friedrich, K., Uthe, F., Jensen, P.O., Moser, C., Nielsen, K.F., Eberl, L., Larsen, T.O., Tanner, D., Hoiby, N., Bjarnsholt, T., and Givskov, M. 2012. Ajoene, a sulfur-rich molecule from garlic, inhibits genes controlled by quorum sensing. *Antimicrobial Agents Chemotherapy*. 56, 2314-2325.
- Jamal, M.A., Rosenblatt, J.S., Hachem, R.Y., Ying, J., Pravinkumar, E., Nates, J.L., Chaftari, A.M., and Raad, I.I. 2014. Prevention of biofilm colonization by gram-negative bacteria on minocycline-rifampin-impregnated catheters sequentially coated with chlorhexidine. *Antimicrobial Agents Chemotherapy*. 58, 1179-1182.
- Jenkins, R., Burton, N., and Cooper, R. 2014. Proteomic and genomic analysis of methicillin-resistant *Staphylococcus aureus* (MRSA) exposed to manuka honey in vitro demonstrated down-regulation of virulence markers. *Journal of Antimicrobial Chemotherapy*. 69, 603-615.
- Ji, G., Beavis, R.C., and Novick, R.P. 1995. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. *Proceeding of the National Academy of Sciences of the United States of America*. 92, 12055-12059.
- Jones, S.M., Morgan, M., Humphrey, T.J., and Lappin-Scott, H. 2001. Effect of vancomycin and rifampicin on methicillin-resistant *Staphylococcus aureus* biofilms. *Lancet*. 357, 40-41.
- Khodavandi, A., Harmal, N.S., Alizadeh, F., Scully, O.J., Sidik, S.M., Othman, F. Sekawi, Z., Ng, K.P., and Chong, P.P. 2011. Comparison between allicin and fluconazole in *Candida albicans* biofilm inhibition and in suppression of HWP1 gene expression. *Phytomedicine*. 19, 56-63.
- Lajcak, M., Heidecke, V., Haude, K.H., and Rainov, N.G. 2013. Infection rates of external ventricular drains are reduced by the use of silver-impregnated catheters. *Acta Neurochirurgica*. 155, 875-881.
- Lauderdale, K.J., Malone, C.L., Boles, B.R., Morcuende, J., and Horswill, A.R. 2010. Biofilm dispersal of community-associated methicillin-resistant *Staphylococcus aureus* on orthopedic implant material. *Journal Orthopaedic Research*. 28, 55-61.
- Lawson, L.D., Wang, Z.J., and Hughes, B.G. 1991. Identification and HPLC quantification of the sulfides and dialk(en)yl thiosulfinates in commercial garlic products. *Planta Medica*. 57, 363-370.
- Lemar, K.M., Turner, M.P., and Lloyd, D. 2002. Garlic (*Allium sativum*) as an anti-*Candida* agent: a comparison of the efficacy of fresh garlic and freeze-dried extracts. *Journal of Applied Microbiology*. 93, 398-405.
- Lihua, L., Jianhui, W., Jialin, Y., Yayin, L., and Guanxin, L. 2013. Effects of allicin on the formation of *Pseudomonas aeruginosa* biofilm and the production of quorum-sensing controlled virulence factors. *Polish Journal of Microbiology*. 62, 243-251.
- Lodise, T.P. and McKinnon, P.S. 2005. Clinical and economic impact of methicillin resistance in patients with *Staphylococcus aureus* bacteremia. *Diagnostic Microbiology and Infectious Disease*. 52, 113-122.
- Makino, T., Jimi, S., Oyama, T., Nakano, Y., Hamamoto, K., Mamishin, K., Yahiro, T., Hara, S., Takata, T., and Ohjimi, H. 2013. Infection mechanism of biofilm-forming *Staphylococcus aureus* on indwelling foreign materials in mice. *International Wound Journal*. doi:10.1111/iwj.12061.
- Miron, T., Rabinkov, A., Mirelman, D., Wilchek, M., and Weiner, L. 2000. The mode of action of allicin: Its ready permeability through phospholipid membranes may contribute to its biological activity. *Biochimica et Biophysica Acta*. 1463, 20-30.
- Nidadavolu, P., Amor, W., Tran, P.L., Dertien, J., Colmer-Hamood, J.A., and Hamood, A.N. 2012. Garlic ointment inhibits biofilm formation by bacterial pathogens from burn wounds. *Journal of Medical Microbiology*. 61, 662-671.
- Novick, R.P. 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Molecular Microbiology*. 48, 1429-1449.
- Otto, M. 2008. Staphylococcal Biofilms. *Current Topics in Microbiology and Immunology*. 322, 207-228.
- Palavecino, E. 2004. Community-acquired methicillin-resistant *Staphylococcus aureus* infections. *Clinics in Laboratory Medicine*. 24, 403-418.
- Perez-Giraldo, C., Cruz-Villalon, G., Sanchez-Silos, R., Martinez-Rubio, R., Blanco, M.T., and Gomez-Garcia, A.C. 2003. In vitro activity of allicin against *Staphylococcus epidermidis* and influence of subinhibitory concentrations on biofilm formation. *Journal of Applied Microbiology*. 95, 709-711.
- Rasmussen, T.B., Bjarnsholt, T., Skindersoe, M.E., Hentzer, M., Kristoffersen, P., Kote, M., Nielsen, J., Eberl, L., and Givskov, M. 2005. Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. *Journal of Bacteriology*. 187, 1799-1814.
- Rattanachaikunsopon, P. and Phumkhachorn, P. 2009. Antimicrobial activity of elephant garlic oil against *Vibrio cholerae* in vitro and in a food model. *Bioscience, Biotechnology, and Biochemistry*. 73, 1623-1627.

- Rewatkar, A.R. and Wadher, B.J. 2013. *Staphylococcus aureus* and *Pseudomonas aeruginosa* - biofilm formation methods. IOSR Journal of Pharmacy and Biological Sciences. 8, 36-40.
- Rezaei, M., Moniri, R., Gholam, S., Mousavi, A., and Shiade, M.J. 2013. Prevalence of biofilm formation among methicillin resistance *Staphylococcus aureus* isolated from nasal carriers. Jundishapur Journal of Microbiology. 6(6): e9601. doi:10.5812/jjm.9601
- Shuford, J.A., Stckelberg, J.M., and Patel, R. 2005. Effect of fresh garlic extract on *Candida albicans* biofilms. Antimicrobial Agents and Chemotherapy. 49, 473.
- Stepanovic, S., Vukovic, D., Dakic, I., Savic, B., and Svabic-Vlahovic, M. 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. Journal of Microbiological Methods. 40, 175-179.
- Thien-Fah, C.M. and O'Toole, G.A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends in Microbiology. 9, 34-39.
- Thomas, J.G. and Lehman, D.C. 2006. Biofilms: architects of disease. In Textbook of Diagnostic Microbiology, Mahon, C., Manuselis, G. and Lehman, D., editors. Elsevier, Amsterdam, Netherlands, pp. 884-895.
- Tran, P.L., Lowry, N., Campbell, T., Reid, T.W., Webster, D.R., Tobin, E., Aslani, A., Mosley, T., Dertien, J., Colmer-Hamood, J.A., and Hamood, A.N. 2012. An organo-selenium compound inhibits *Staphylococcus aureus* biofilms on hemodialysis catheters in vivo. Antimicrobial Agents and Chemotherapy. 56, 972-978.
- Tsao, S.M. and Yin, M.C. 2001. In-vitro antimicrobial activity of four diallyl sulphides occurring naturally in garlic and Chinese leek oils. Antimicrobial Agents and Chemotherapy. 50, 646-649.
- Tsao, S.M., Hsu, C.C., and Yin, M.C. 2003. Garlic extract and two diallyl sulphides inhibit methicillin-resistant *Staphylococcus aureus* infection in BALB/cA mice. Journal of Antimicrobial Chemotherapy. 52, 974-980.
- Viswanathan, V., Phadatare, A.G., and Mukne, A. 2014. Antimycobacterial and Antibacterial activity of *Allium sativum* bulbs. Indian Journal of Pharmaceutical Sciences. 76, 256-261.
- Widmer, A.F. 2001. New developments in diagnosis and treatment of infection in orthopedic implants. Clinical Infectious Diseases. 33, S94-106.
- Wu, X., Santos, R.R., and Fink-Gremmels, J. 2015. Analyzing the antibacterial effects of food ingredients: model experiments with allicin and garlic extracts on biofilm formation and viability of *Staphylococcus epidermidis*. Food Science and Nutrition. 3, 158-168.
- Yarwood, J.M., Bartels, D.J., Volper, E.M., and Greenberg, E.P. 2004. Quorum sensing in *Staphylococcus aureus* biofilms. Journal of Bacteriology. 186, 1838-1850.
- Zhai, H., Pan, J., Pang, E., and Bai, B. 2014. Lavage with allicin in combination with vancomycin inhibits biofilm formation by *Staphylococcus epidermidis* in a rabbit model of prosthetic joint infection. PLOS ONE. 9, e102760. doi:10.1371/journal.pone.0102760.